Viable and culturable populations of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and *Starmerella bacillaris* (synonym *Candida zemplinina)* during Barbera must fermentation

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Chunxiao Wang[a], Braulio Esteve-Zarzoso[a], Luca Cocolin[b], Albert Mas[a], Kalliopi Rantsiou[b,*]

[a]Departament de Bioquímica i Biotecnologia, Facultat d' Enologia, Universitat Rovira i Virgili, Marcel·lí Domingo 1, Tarragona 43007, Spain.

[b]Dipartimento di Scienze Agrarie, Forestali e Alimentari, Università degli studi di Torino, Largo Paolo Braccini 2, Grugliasco 10095, Italy.

[*] Corresponding author:

Kalliopi Rantsiou

Dipartimento di Scienze Agrarie, Forestali e Alimentari, Università degli studi di Torino, Largo Paolo Braccini 2, Grugliasco 10095, Italy.

Tel 39 011 670 8870, Fax 39 011 670 8549, E-mail: kalliopi.rantsiou@unito.it.

ABSTRACT

The present study analyzed the viable and/or culturable populations of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and *Starmerella bacillaris* (synonym *Candida zemplinina*) during laboratory grape must fermentation, in order to investigate the interaction between the three species considered. Firstly, population dynamics during wine fermentation were followed by culture-dependent techniques, and non-*Saccharomyces* yeast became non-culturable at late stages of fermentation when *S. cerevisiae* dominated. Four different culture-independent techniques were further applied to detect viable yeast cells at the late stage of fermentation. Both quantitative PCR techniques applied, namely ethidium monoazide bromide (EMA)-qPCR and Reverse Transcription (RT)-qPCR, detected *H. uvarum* and *Starm. bacillaris* at a concentration of 10^5 to 10^6 cells/mL. These non-culturable cells had membranes impermeable to EMA and stable rRNA. The background signals from dead cells did not interfere with the quantification of viable cells in wine samples by EMA-qPCR technique. As a qualitative culture-independent technique, DGGE technique was coupled with EMA treatment (EMA-PCR-DGGE) or with RT (RT-PCR-DGGE). With EMA-PCR-DGGE non-*Saccharomyces* species during fermentation were detected although it was limited by the predominance of *S. cerevisiae*.

Keywords: culture-independent technique, DGGE, ethidium monoazide bromide (EMA), quantitative PCR (qPCR), wine, non-*Saccharomyces*.

1 Introduction

Wine fermentations are complex ecological processes with a succession of variable yeast species that could provide different characteristics to the fermentation and final wine (Fleet, 2003). Considerable efforts have been devoted to investigate and monitor population dynamics during mixed fermentations with *Saccharomyces* and non-*Saccharomyces* species. According to culture-dependent analysis, the non-*Saccharomyces* species are only isolated from early stages and the fermentations are dominated by *Saccharomyces* at late stages (Fleet, 2008). Recently, using culture-independent techniques, it was highlighted that viable non-*Saccharomcyes* populations could be quantified at late stages of fermentation (Andorrà et al., 2008; Wang et al., 2014) supporting their possible role also at the end of the transformation process. For these reasons, a thorough study is required to understand the states (culturable, live, injured or dead) of non-*Saccharomyces* during fermentation.

The application of culture-independent techniques in wine ecology studies is considered a valid approach to investigate the presence of viable but non-culturable (VBNC) cells (Cocolin et al., 2013). For the quantitative techniques, qPCR was firstly used to directly quantify yeast DNA from wine samples (Hierro et al., 2007; Tofalo et al., 2012), but because of the stability of DNA, dead cells were also quantified resulting in an overestimation of yeast populations. To solve this problem, RT-qPCR, fluorescence in situ hybridization (FISH) and qPCR using ethidium monoazide bromide treatment or propidium monoazide bromide (EMA-qPCR or PMA-qPCR) were explored (Hierro et al., 2006; Rawsthorne and Phister, 2009; Andorrà et al., 2010a; Shi et al., 2012; Wang et al., 2014; Vendrame et al., 2014). In RT-qPCR and FISH the cells with rRNA are considered viable, because the rRNA is less stable than DNA and, thus, is not quantified in dead cells (Hierro et al., 2006, Andorrà et al., 2011, Wang et al., 2014). EMA-qPCR excludes cells with compromised membranes; EMA enters these cells and covalently combines with DNA which is not amplified by subsequent

PCR reactions (Rudi et al., 2005). All of these quantitative techniques require specific primers, which increase the sensitivity for detection, avoiding the detection of non-targeted yeast species. As qualitative techniques, PCR-DGGE and RT-PCR-DGGE have been developed with universal primers to detect all probable yeast species without the need to know their sequences (Cocolin et al., 2000; Mills et al., 2002). However, detection sensitivity of DGGE depended on the disparity of orders of magnitude among different populations (Mills et al., 2002; Andorrà et al., 2008; Cocolin et al., 2011). Therefore, to analyze the yeast species in complicated must samples during fermentations, it is better to use both qualitative and quantitative techniques.

This study focused on three main species in must fermentations: *Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and *Starmerella bacillaris* (synonym *Candida zemplinina*), with the aim to analyse the vitality state of the cells during fermentation, especially at late stages. These two non-*Saccharomyces* species were studied here because of their common appearance on Barbera grape in Piedmont region of Italy (Alessandria et al., 2015). The population dynamics during the whole fermentation was monitored by culture-dependent techniques. When the cell culturability was lost for the non-*Saccharomyces* species, EMA-qPCR and EMA-PCR-DGGE, as well as RT-qPCR and RT-PCR-DGGE were used to determine the cells' status in wine samples. Dead cells, after 75% ethanol treatment, and culturable cells were used as negative and positive controls, respectively, to raise the standard curves for qPCR techniques and markers for DGGE.

2 Material and methods

2.1 Yeast strains

H. uvarum Y1 (Mills et al., 2002) and *Starm. bacillaris* CBE4 (Englezos et al., 2015) were obtained from yeast culture collection of the DISAFA (Dipartimento di Scienze Agrarie,

Forestali e Alimentari, University of Torino, Italy). Uvaferm BC (*S. cerevisiae*) was obtained from Lallemand (Montreal, Canada). The yeast cultures from DISAFA were grown on YPD agar (1% yeast extract, 2% peptone, 2% dextrose, all from Biogenetics, Italy), and then were inoculated into 10 mL of sterile Barbera must for a preadaptation. The Uvaferm BC was activated following the suppliers' instructions and subsequently preadapted in the same must.

2.2 Wine fermentation and sampling

Red Barbera grape berries were harvested in 2013 and after crushing grape must was stored at -20 °C. Before use, the grape must was defrosted at 4 °C, and then pasteurized at 65 °C for 1 h. Flasks of 250 ml containing 100 mL of Barbera must (Glucose+Fructose 234.00 g/L, malic acid 3.8 g/L, citric acid 0.3 g/L, tartaric acid 5.8 g/L, pH 2.95, and YAN 179.60 mg N/L) were inoculated with 1 x 10^5 cells/mL of preadapted Uvaferm BC, *Starm. bacillaris* CBE4 and *H. uvarum* Y1. Fermentations were performed in duplicate at 25 °C, statically in presence of air (with screw cap not totally tight), and the whole process was monitored by weight every 24 h until no further weight loss. Samples (3 mL) were taken at day 0, 1, 4, 6, 8, 11 and 14. One mL sample was used for microbiological analysis and another two tubes with 1 mL sample were centrifuged at 14,000 rpm for 10 min. The supernatants were collected for analysis of main parameters using an HPLC (Agilent Technologies 1260 Infinity, USA) according to Giordano et al. (2009), and the content of YAN was measured by L-arginine / urea / ammonia assay kit (Megazyme, Ireland) and primary amino nitrogen assay kit (Megazyme, Ireland). The pellet in one tube was passed to EMA treatment and further DNA extraction, and the pellet in the other tube was suspended in 100 μL of RNA*later*® solution (Ambion, USA), and then kept at -20 °C for further RNA extraction.

2.3 Microbiological analysis

Appropriate dilutions in ten-fold series by Ringers solution (Oxoid, Italy) from 1 mL of must were spread onto WL nutrient agar (Biogenetics) and Lysine medium (Oxoid). Counting

was done after five days' growth at 28°C. Colonies of the three yeast strains were discriminated by different morphologies and colors on WL nutrient agar (Cavazza et al., 1992). Lysine medium was used for quantification of non-*Saccharomyces* population when *S. cerevisiae* dominated the fermentation.

2.4 EMA treatment and DNA extraction

The cells' pellet from 1 mL of sample was resuspended in 1 mL of YPD broth and kept at 13 °C for 2 h to recover cells' membrane from the ethanol interference (Andorrà et al., 2010a). Then cells were collected, suspended in 1 mL of sterile water and treated with EMA (Sigma-Aldrich, Italy) solution (5 g/L) using the same device and procedure as described by Andorrà et al. (2010a). Briefly, EMA was dissolved in sterile Milli-Q water to prepare the solution of 5 g/L. The box with a 650-W halogen lamp was constructed, and the distance between the lamp and the tube was approx. 20 cm. Two microliters of EMA solution were added to the cell suspension (final concentration of EMA was 24 μM), and incubated in dark for 10 min. The samples were exposed to light for 30 s, kept on ice for 1 min with light off, and exposed again to light for 30 s. Cells were collected by centrifugation and washed with 1 mL of sterile water to remove the unstained EMA. Masterpure™ Complete DNA & RNA Purification kit (Epicentre, USA) was used to extract DNA according to the manufacturer's instruction. The DNA extracted was conserved at -20 ºC and used for both EMA-qPCR and EMA-PCR-DGGE analysis.

2.5 RNA extraction and reverse transcription

The cell suspension preserved in RNA*later*® solution was centrifuged, and RNA from cell pellet was extracted by Masterpure™ Complete DNA & RNA Purification kit (Epicentre, USA) following manufacturer's instructions. RNA was treated with TURBO™ DNase (Ambion, USA) at 37 °C for 3 h or overnight (if needed) to completely remove DNA. Complete DNA removal was confirmed by qPCR. Reverse transcription of the extracted RNA

was performed with the following procedure: 0.5 μL of RNA was mixed in 4.5 μL of DNase and RNase-free water containing 0.5 μL of Random Primers (500 μg/mL, Promega, Italy), and incubated at 72 °C for 5 min. The reaction sample was kept on ice for 5 min, and then added with 7.5 μL of mixture containing 2.5 μL of M-MLV RT 5× Buffer, 2.5 μL of 10mM dNTPs, 20 U of RNase inhibitor (all from Promega, Italy), 100 U of M-MLV Reverse transcriptase (Promega), and 1.5 μL of DNase and RNase-free water. The reaction continued with incubation at 42 °C for 1 h and stopped with a step of 72 °C for 10 min. The cDNA synthesized was conserved at -20 ºC and used for further RT-qPCR and RT-PCR-DGGE analysis.

2.6 Standard curves and qPCR analysis

The qPCR was performed with the primers (all from Sigma-Aldrich, Italy) YEASTF/YEASTR for total yeast (Hierro et al. 2006), CESPF/SCERR for *S. cerevisiae* (Hierro et al. 2007), CESPF/HUVR for *H. uvarum* (Hierro et al. 2007), and AF/200R for *Starm. bacillaris* (Andorrà et al. 2010b). Each reaction was carried out by the MiniOpticon™ Real-Time PCR System (Bio-Rad, Italy) in a total volume of 13 μL of reaction mixture, which contained 6 μL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 0.4 μM of the primers, and 1 μL of DNA or cDNA template. PCR conditions were as in Andorrà et al. (2010b). Standard curves of EMA-qPCR and RT-qPCR were constructed for each yeast species in triplicate using 10-fold serial dilutions of preadapted cells. These cells were obtained from one day's yeast cultures in 10 mL of sterile Barbera juice as mentioned in 2.1. The serial dilutions were performed using 9 mL of sterile must to which 1 mL of cell suspension was added. The final cell suspensions were counted by hemocytometer under microscope to determine the real concentrations of serial cell dilutions.

Dead cells were prepared from the same cultures, and then treated with 75% ethanol for 24 h. The lysis process of cells was confirmed by absence of growth in YPD broth after 24 h and on WL nutrient agar after 5 days.

2.7 PCR and DGGE analysis

Primers $NL1^{GC}$ and LS2 were used to amplify the ribosomal region of extracted DNA or synthesized cDNA for further DGGE analysis (Mills et al., 2002). Five μL of the PCR products were firstly checked for the sizes by agarose gel electrophoresis, then, the same volume was further separated by DGGE gel electrophoresis in a DCode universal mutation detection system (Bio-Rad). Both kinds of electrophoresis were operated and the gels were stained and photographed according to the descriptions of Mills et al. (2002) with minor modifications: DGGE gel used a denaturing gradient from 30% to 60% of urea and formamide, and was run at 120 V for 4 h. PCR products from DNA or cDNA of pure yeast cultures were used as markers in DGGE gel. Different cell mixtures were prepared from pure yeast cultures, and subjected to EMA-PCR-DGGE analysis to determine the detection limits of the three species.

2.8 Data analysis

Statistical analyses of variations were performed by One-Way ANOVA to calculate the value of F and significance, with post-hoc Tukey test when needed, using IBM SPSS Statistics 23. The Ct values from live and dead cells were used directly for variation analysis, and the yeast population numbers analyzed by different techniques were converted to logarithm value for further variation analysis.

3 Results

3.1 Culturable yeast populations during must fermentation

The fermentations terminated after eleven days, although culturability analysis was extended up to 14 days. During the whole fermentation process, culturable populations of the three species showed different trends, especially at late stages of fermentations (Fig. 1). The three species grew to population of 10^7 colony forming units (cfu)/mL during the first days of fermentation. *S. cerevisiae* (Uvaferm BC) maintained the maximum population level during fourteen days. *Starm. bacillaris* CBE4 kept similar population level as *S. cerevisiae* during eight days, decreasing sharply to undetectable levels by plating on day 11. The other non-*Saccharomyces* species, *H. uvarum* Y1, grew faster to reach a population of 10^7 cfu/mL, but decreased to undetectable level earlier (day 6) than the *Starm. bacillaris* CBE4 strain.

3.2 qPCR analysis of wine samples

First, standard curves for each species and both techniques (EMA and RT- qPCR) were separately constructed. Background signal from dead cells at different concentrations were also quantified. Finally, the populations of each species in the selected samples were quantified and compared among the two culture-independent techniques and the culture-dependent techniques.

3.2.1 Standard curves for EMA-qPCR

Cells adapted in sterile Barbera must were serially diluted in the same must and quantified by microscope to associate the logarithm values of cells' concentration and Ct values. Good correlations were obtained for populations between 10^3 and 10^7 cells/mL by EMA-qPCR analysis (Tab. 1).

The preadapted cells were also used to prepare dead cells, and the dead cells of three species were analyzed by EMA-qPCR technique to quantify the background. The Ct values from dead cells were much higher when compared to those generated by live cells at the same cell concentration (Tab. 2). The ANOVA analysis demonstrated the significant difference of Ct values between live and dead cells due to the lower significance value than 0.05, despite

that no difference was observed for *Starm. bacillaris* at the concentration of 10^3 cells/mL (the significance value > 0.05), most likely due to the high difference of Ct values within live and dead cells. Interference from background signal appeared only in presence of large populations of dead cells (10^7 cells/mL), which were detected by the method as approx. 10^4 live cells/mL according to the standard curves.

3.2.2 Standard curves of RT-qPCR

Good correlations were also obtained for culturable populations between 10^2 and 10^7 cells/mL by RT-qPCR analysis (Tab. 1). Ct values of *S. cerevisiae*, *H. uvarum*, and *Starm. bacillaris* were also checked before and after the killing treatment (75% ethanol for 24h) and approx. 10^4 to 10^5 cells/mL background was produced from 10^6 to 10^7 dead cells/mL of each species according to the standard curve in Table 1 (data not shown).

3.2.3 Analysis of fermentation samples

The DNA and cDNA extracted from fermentation samples were subjected to amplification and specific yeast populations were quantified using the previously generated standard curves. More specifically, samples at day 6 and 11, in which the *H. uvarum* and *Starm. bacillaris* respectively became non-culturable, were analyzed. The chemical composition of the fermented must samples is shown in Table 3.

The EMA-qPCR and RT-qPCR quantification results were compared to those obtained by culture-dependent techniques (Tab. 4). The ANOVA analysis did not differentiate among three techniques for the total yeast and *S. cerevisiae* quantification on day 6, but some differences existed for the other samples. The post-hoc Tukey test was further performed to find out the culture-independent technique, by which the quantification result was significantly different from the result by culture-dependent techniques, as shown in Table 4. Therefore, three main conclusions could be obtained. First of all, the total yeast populations from all samples were kept at similar level (10^7 cells/mL) by culture-dependent and

culture-independent techniques because of the existence of large culturable *S. cerevisiae* population. Secondly, when the culturable species were quantified on plates, the population size was similar to the one detected by EMA-qPCR, both for *S. cerevisiae* and *Starm. bacillaris*. However, quantification by RT-qPCR yielded counts that were one log unit lower for *S. cerevisiae* and even two log units lower for *Starm. bacillaris* comparing to culture-dependent methods. Then, when no colonies of non-*Saccharomyces* were recovered on plates, still populations of about 10^5 cells/mL (*H. uvarum*) or 10^6 cells/mL (*Starm. bacillaris*) were quantified by EMA-qPCR. The counts of the non-culturable non-*Saccharomyces* by RT-qPCR were similar to those obtained by EMA-qPCR.

3.3 DGGE electrophoretic profiles of wine samples

In order to study the effect of the EMA treatment on the detection of the three species by DGGE, eight different cell mixtures were prepared from pure yeast cultures in sterile Barbera juice (Fig. 2). The differences in population sizes affected the detection and when *S. cerevisiae* was predominant in the mixture with 10^7 cells/mL, *Starm. bacillaris* could be detected at 10^5 cells/mL but not at 10^3 cells/mL, whereas *H. uvarum* was not detected in any of those tested concentrations. When low populations of *S. cerevisiae* were present in the mixture (10^3 cells/mL), *Starm. bacillaris* at 10^5 and 10^7 cells/mL could be detected, while *H. uvarum* could only be seen at concentrations of 10^7cells/mL or when *Starm. bacillaris* was at the same concentration (10^5 cells/mL).

The fermentation samples (day 6 and 11) were also analyzed by the EMA-PCR-DGGE and RT-PCR-DGGE techniques. The results are shown in Fig. 3. With EMA-PCR-DGGE, *S. cerevisiae* was detected in both tested days (6 and 11), *Starm. bacillaris* was only found on day 6 while *H. uvarum* was not detected. Although these results were similar to plating, the differences in population sizes between all the species affected clearly the detection of the minority species (*H. uvarum* and *Starm. bacillaris*).

Analysis by RT-PCR-DGGE yielded very different results. *S. cerevisiae*, *H. uvarum* and *Starm. bacillaris* could be detected in all samples although *H. uvarum* presented very strong bands.

4 Discussion

The definition of live cells in wine ecology has changed with the development of detection techniques. Starting from traditional methods (i.e. use of culture media), analysis has moved to the application of culture-independent molecular techniques represented by the use of DNA (qPCR or PCR-DGGE), determination of cell membrane integrity (EMA-qPCR or PMA-qPCR) or use of RNA (RT-qPCR, RT-PCR-DGGE and FISH) (Cocolin and Mills, 2003; Hierro et al., 2006; Andorrà et al., 2008; Andorrà et al., 2010a; Andorrà et al., 2010b; Shi et al., 2012; Wang et al., 2014; Vendrame et al., 2014). The combined use of culture-dependent and culture-independent techniques was considered in this paper, and the aim was to study how the interactions between *S. cerevisiae*, *H. uvarum* and *Starm. bacillaris* during alcoholic fermentation of a natural must could be reflected at population level. A need for truly dead cells was necessary for comparison and background estimation. Heat shock (65ºC) and ethanol toxicity (75%) were tested by reactivation in both rich medium (YPD broth) and differential media (WL nutrient agar). Heat shock and ethanol toxicity yielded the same results (data not shown). Ethanol toxicity was chosen for the similarity to the increased concentrations of ethanol during alcoholic fermentations, which could finally produce cell death.

The agreement between culture-dependent techniques and EMA-qPCR for culturable *S. cerevisiae* and *Starm. bacillaris* cells was considered as a proof that culturable cells had fully functional cell membrane and EMA-qPCR could quantify them accurately, as previously described by Andorrà et al (2010a). EMA-qPCR presented good linearity with culturable

populations between 10^3 to 10^7 cells/mL, but a low background signal was produced from the dead cells at high concentrations. This background signal was also observed by Andorrà et al. (2010a) and Nkuipou-Kenfack et al. (2013), and it could not be removed by regulation of EMA treatment conditions (Nkuipou-Kenfack et al., 2013). Nevertheless, from the view of application, this background signal did not actually interfere with the quantification of live cells from wine fermentations, because the signal (10^5 to 10^6 cells/mL) was always higher than background signals (10^4 cells/mL). This point was also verified by Andorrà et al. (2010a), by addition of a constant population of dead cells to serial dilutions of viable cells obtaining a standard curve that was not influenced by the dead cells.

The analysis of rRNA integrity through the RT-qPCR analysis resulted in an underestimation of the culturable population by one or two log units. Considering the good linearity of the standard curves, this result is probably related with a decreased ribosome level inside the culturable cells facing environmental stress (ethanol production, nutrient depletion) and initiating survival strategies. Although there is no evidence in the present work, other studies based on FISH (Andorrà et al., 2011; Wang et al., 2014) also observed the variation in the fluorescence intensity of the stained *S. cerevisiae* cells during fermentation. The variation of rRNA concentration to some extent questioned the quantification accuracy of RT-qPCR, especially as reference for live cells. Furthermore, the rRNA of dead cells might be degraded at different rates depending on the lytic process. By ethanol treatment (75% ethanol for 24h), the reduction of rRNA was obvious, although some stable rRNA still existed after 48h (data not shown) and probably interfered with the quantification of live cells. Previous data from dead cells originated by heat shock (60 °C 20 min, Hierro et al., 2006) also showed the relative stability of rRNA, which takes at least 24h for significant degradation in *S. cerevisiae* cells. The death of non-*Saccharomyces* species during wine fermentation is probably dependent on a variety of factors (Wang et al., 2015) and their effect on the relative stability

of rRNA in these dying cells is still far from being understood. More information is needed to understand the process, and the use of rRNA-dependent techniques to evaluate it.

Previous studies based on culture-independent techniques have reported the existence of non-*Saccharomyces* populations during late stages of fermentation, when *Saccharomyces* dominated the process (Andorrà et al., 2010a; Wang et al., 2014). Our results were consistent with these reports because of the detection of 10^5 to 10^6 cell/mL of *H. uvarum* and *Starm. bacillaris* after no culturable cells were obtained from these species. Based on these results, we can conclude that a subpopulation of non-culturable cells had an injured membrane (therefore were not detected by EMA-qPCR) and are considered dead while a quantifiable number of non-culturable cells were still alive with functional membranes (detected by EMA-qPCR) and non-degraded RNA (detected by RT-qPCR).

DGGE was firstly used in this study with combination of EMA treatment, and the approximate detection limits for *Starm. bacillaris* and *H. uvarum* were also tested. It could be used as a basic qualitative culture-independent technique for monitoring wine fermentation. The limitation of EMA-PCR-DGGE was the uncertain detection of minor populations when some predominant populations existed at one or two log units higher concentrations. RT-PCR-DGGE in this study detected all of the three species at late stages of fermentation, especially from *H. uvarum*, which was the species undetected on plates. The high intensity of rRNA signal from *H. uvarum* could be interpreted as a high concentration of rRNA, as seen before (Andorrà et al., 2011) on a strain of *Hanseniaspora guilliermondii*. However, the application of this technique to follow live cells during wine fermentation needs the support of further data due to the unclear rRNA relative stability in dead cells which is also probably species-dependent.

In conclusion, the present work detected viable but non-culturable *H. uvarum* and *Starm. bacillaris* cells by culture-independent techniques. These cells presented functional

membranes and non degraded rRNA. Also both *S. cerevisiae* and *Starm. bacillaris* presented cell membrane integrity, relatively stable rRNA and culturability during late stages of grape must fermentation. The comparative analysis among different techniques demonstrated the potential of EMA-qPCR and EMA-PCR-DGGE for wine ecological studies. This work also indicates some underlying obstacles for the application of RT-qPCR and RT-PCR-DGGE on the estimation of viable populations of different species during alcoholic fermentation. The relative stability of rRNA during the process of cell lysis needs to be determined with precision before being applied systematically for routine analysis of viable populations in alcoholic fermentations.

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445 Table 1. The slope, intersection, correlation coefficient (R^2) and efficiency of standard curves
446 of total yeast, *S. cerevisiae*, *H. uvarum*, and *Starm. bacillaris* by EMA-qPCR analysis and
447 RT-qPCR analysis. The efficiency was calculated by the formula $10^{-1/slope}$ -1. Mean and
448 standard deviation of triplicate qPCR amplifications are shown.

Technique	Yeast	Slope	Intersection	R^2	Efficiency (%)
EMA-qPCR	Total yeast	-2.8250±0.2490	36.924±0.813	0.9943±0.0061	125.93±23.17
	S. cerevisiae	-3.4097±0.0807	42.319±0.484	0.9909±0.0005	96.46±3.21
	H. uvarum	-3.2230±0.0198	38.599±0.103	0.9976±0.0037	104.30±0.89
	Starm. bacillaris	-3.8530±0.3224	47.819±2.024	0.9912±0.0152	81.78±9.06
RT-qPCR	Total yeast	-2.4045±0.0770	30.095±0.3734	0.9796±0.0021	160.55±8.00
	S. cerevisiae	-2.9293±0.0741	40.454±0.479	0.9870±0.0009	119.47±4.39
	H. uvarum	-3.1147±0.1020	41.340±0.4815	0.9964±0.0030	109.44±5.21
	Starm. bacillaris	-3.3408±0.0364	37.036±0.3539	0.9923±0.0006	99.22±1.48

449

450 Table 2. Ct values for a dilution series of live or dead cells by EMA-QPCR. Cell
451 concentration is expressed as log units, and Ct values were shown as mean ± standard
452 deviation of triplicate qPCR amplifications. The significance level for One-Way ANOVA
453 calculation was 0.05.
454

Species	Cell concentration	Live	Dead	Variation between live & dead cells	Variation within live & dead cells	F	Significance
S. cerevisiae	7	17.89±0.04	28.76±0.38	117.61	0.07	1599.65	0.001
	6	22.70±0.00	29.23±0.21	42.60	0.02	1893.26	0.001
	5	25.12±0.16	31.65±0.04	44.09	0.01	3391.51	<0.001
	4	28.70±0.17	31.81±0.01	14.87	0.01	1020.81	0.001
	3	31.94±0.35	34.19±0.01	4.00	0.06	63.92	0.015
H. uvarum	7	16.25±0.10	24.39±0.09	66.34	0.01	7270.25	<0.001
	6	18.98±0.08	30.87±0.19	141.25	0.02	6472.08	<0.001
	5	22.33±0.16	31.53±0.59	84.82	0.19	457.40	0.002
	4	26.01±0.34	32.98±0.18	48.58	0.07	652.09	0.002
	3	28.85±0.21	32.95±0.82	16.77	0.36	45.98	0.021
Starm. bacillaris	7	20.83±0.09	30.63±0.16	97.42	0.02	5582.63	<0.001
	6	24.14±0.01	33.95±0.74	96.33	0.27	356.23	0.003
	5	29.15±0.25	35.21±0.46	31.08	0.14	225.18	0.004
	4	32.97±0.01	37.49±0.50	20.43	0.13	162.08	0.006
	3	35.68±0.71	38.00±0.01	5.41	1.46	3.69	0.195

455 Table 3. Chemical composition of grape must main components at different fermentation
456 stages. The values are means of duplicate analysis.

Day	Glucose g/L	Fructose g/L	Ethanol % vol	Glycerol g/L	Acetic acid g/L
0	116.60	117.40	0.00	0.10	0.00
6	24.44	34.22	10.00	6.94	0.12
11	0.27	1.09	13.99	8.07	0.26

457

458 Table 4. Yeast quantification on day 6 and 11 by culture-dependent (microbiological analysis)
459 and culture-independent techniques (EMA-qPCR and RT-qPCR). The values of populations in
460 the table are the average from duplicate fermentation and expressed as cfu/mL (plate counting)
461 or cells/mL (qPCR methods), nd means not detectable. The significance level for One-Way
462 ANOVA calculation was 0.05. * The mean difference was significant from culture-dependent
463 technique by post-hoc Tukey test.

Day	Yeast	WL and LM plates	EMA-qPCR	RT-qPCR	Variation between techniques	Variation within techniques	F	Significance
6	Total yeast	5.71×10^{7}	2.08×10^{7}	1.08×10^{8}	0.28	0.04	6.45	0.082
	S. cerevisiae	3.03×10^{7}	2.79×10^{7}	6.03×10^{6}	0.30	0.03	9.20	0.053
	H. uvarum	nd	3.16×10^{5} *	9.10×10^{5} *	21.67	0.06	392.29	<0.001
	Starm.bacillaris	2.68×10^{7}	1.52×10^{7}	5.40×10^{5} *	1.72	0.03	60.81	0.004
11	Total yeast	3.50×10^{7}	1.41×10^{7}	1.08×10^{8}	0.40	0.01	66.69	0.004
	S. cerevisiae	3.50×10^{7}	2.60×10^{7}	4.33×10^{6} *	0.48	0.02	36.61	0.008
	H. uvarum	nd	6.50×10^{5} *	4.28×10^{5} *	21.84	<0.01	28285.08	<0.001
	Starm.bacillaris	nd	1.88×10^{6} *	4.42×10^{5} *	23.39	0.07	338.84	<0.001

464

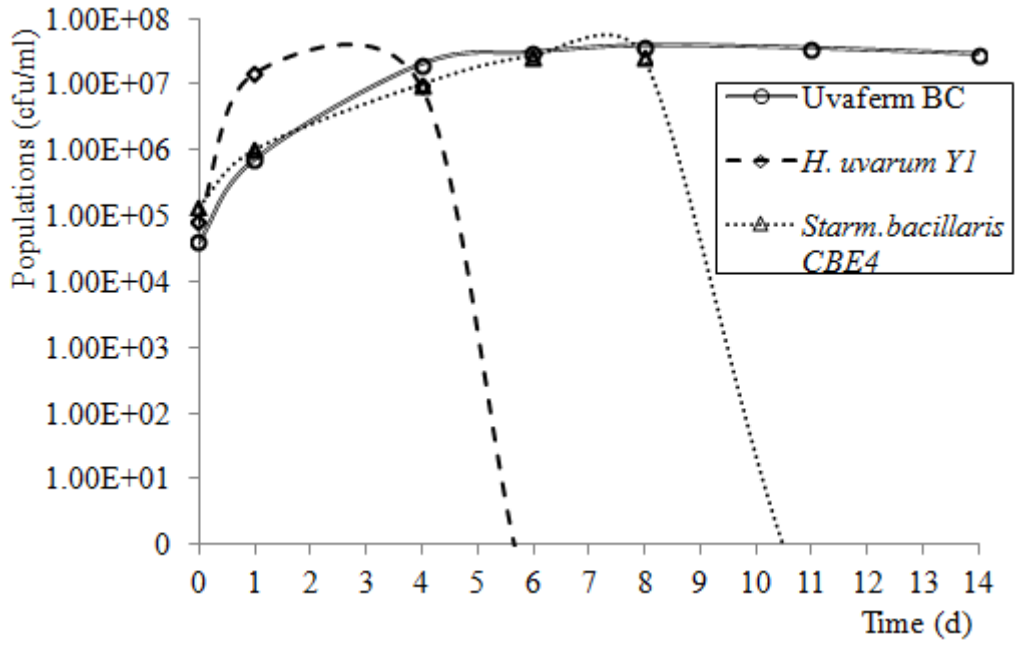

Figure 1. Culturable *S. cerevisiae* (Uvaferm BC), *H. uvarum*, and *Starm. bacillaris* populations during grape must fermentation as determined by plating on WL nutrient agar and Lysine medium. The values are the mean of duplicates.

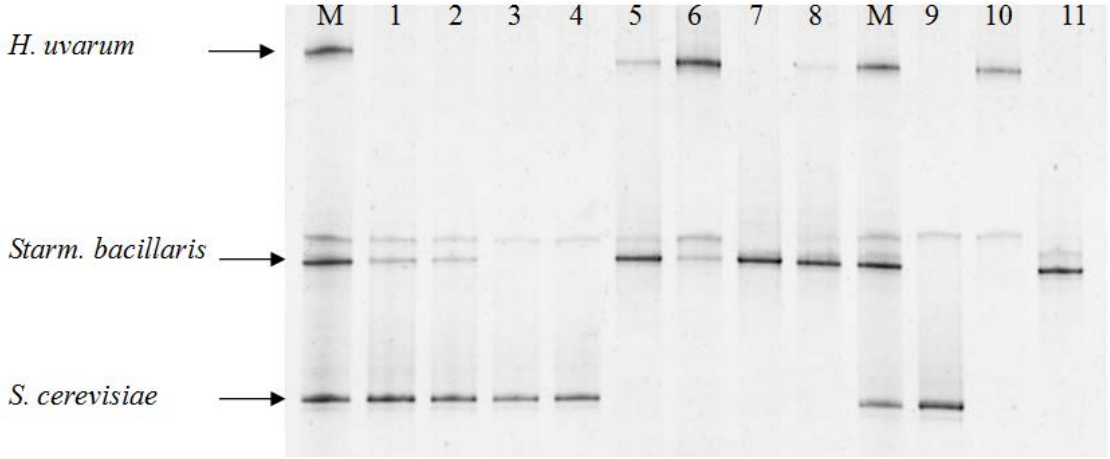

Figure 2. Detection of viable cells from eight different mixtures of *S. cerevisiae* (sacc), *H. uvarum* (huv) and *Starm. bacillaris* (star) by EMA-PCR-DGGE. M, DNA marker made with the three pure species; Lanes 1-11 represent samples of different cell and population mixtures: 1, 10^7sacc+10^3huv+10^5star; 2, 10^7sacc+10^5huv+10^5star; 3, 10^7sacc+10^3huv+10^3star; 4, 10^7sacc+10^5huv+10^3star; 5, 10^3sacc+10^7huv+10^7star; 6, 10^3sacc+10^7huv+10^5star; 7, 10^3sacc+10^5huv+10^7star; 8, 10^3sacc+10^5huv+10^5 star; 9, 10^7sacc; 10, 10^7huv; 11, 10^7star.

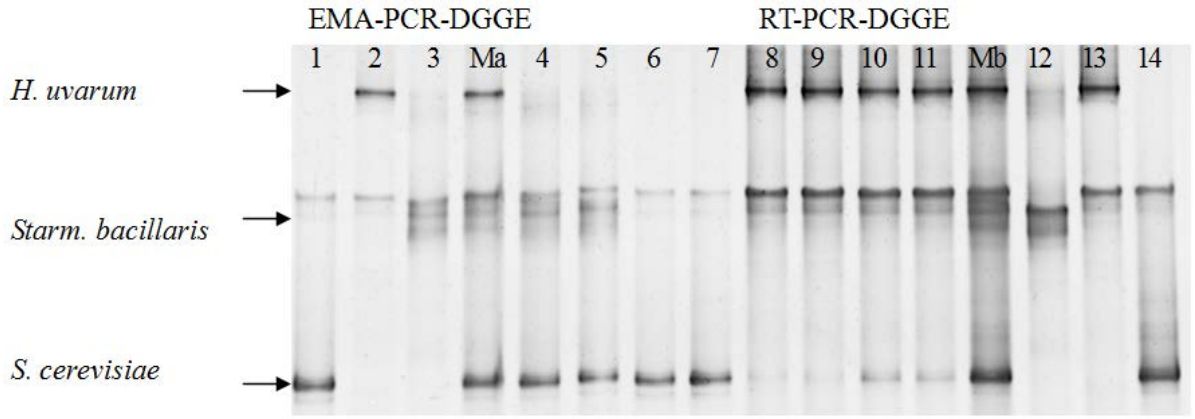

Figure 3. Detection of viable cells of samples from grape must fermentation by EMA-PCR-DGGE and RT-PCR-DGGE analysis. Ma, marker containing mixed DNA of three pure species; Mb, marker containing mixed cDNA of three pure species; Lanes 1-14 were obtained with DNA (1-7) or cDNA (8-14) templates from different samples: 1 and 14, 10^7 *S. cerevisiae*; 2 and 13, 10^7 *H. uvarum*; 3 and 12, 10^7 *Starm. bacillaris*; 4, 5, 8 and 9, samples of day 6 from duplicate fermentations; 6, 7, 10 and 11, samples of day 11 from duplicate fermentations.